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Association of *VSNL1* with schizophrenia, frontal cortical function, and biological significance for its gene product as a modulator of cAMP levels and neuronal morphology

KH Braunewell[1,2,4], AD Dwary[1,4], F Richter[2,4], K Trappe[2], C Zhao[2], I Giegling[3], K Schönrath[2] and D Rujescu[3]

We report an association of single-nucleotide polymorphisms (SNPs) for the *VSNL1* gene (visinin-like 1) with schizophrenia and frontal cortical function in a sample of patients with Diagnostic and Statistical Manual of Mental Disorder-IV (DSM-IV) diagnoses of schizophrenia, compared with healthy controls. Moreover, *VSNL1* SNPs were associated with performance in the Wisconsin Card Sorting Test, a measure for the assessment of frontal cortical function. The *VSNL1* gene product, Visinin-like-protein-1 (VILIP-1), is a member of the neuronal EF-hand Ca^{2+}-sensor protein family. Previously, VILIP-1 mRNA and protein expression were shown to be altered in animal models and in schizophrenia patients. VILIP-1 influences cytosolic cyclic adenosine mono phosphate (cAMP) levels, cell migration, exocytotic processes and differentiation in the periphery. This raises the question, whether, similar to other potential schizophrenia susceptibility genes such as *Disc1*, *PDE4B* and *Akt*, *VSNL1* may affect cAMP signaling and neurite outgrowth in neurons. In dissociated rat hippocampal neurons, VILIP-1 small interfering RNA knockdown decreased cAMP levels and reduced dendrite branching, compared with control-transfected cells. In contrast, VILIP-1 overexpression had the opposite effect. Similar results have been obtained in the human dopaminergic neuronal cell line SH-SY5Y, where the effect on neurite branching and length was attenuated by the adenylyl cyclase inhibitor 2′,5′-dideoxyadenosine and the protein kinase A inhibitor KT5720. These results show that the association of *VSNL1* SNPs with the disease and cognitive impairments, together with previously observed pathological changes in VILIP-1 protein expression, possibly occurring during brain development, may contribute to the morphological and functional deficits observed in schizophrenia.
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Introduction

Schizophrenia is a major disabling psychiatric disorder affecting approximately 1% of the population worldwide. The developmental disease originates from disturbances in very complex combination of genetic information, as well as environmental factors.[1,2] About 2.2 million US citizens are diagnosed with schizophrenia, which, due to its early onset, results in an estimated cost for society of approximately $60 billion annually.[3] Pathological findings, including reduced interneuronal space and dendritic differentiation, decrease in dendritic markers, and a reduction in dendritic length,[4–7] provide neuroanatomical evidence for the observed aberrant connectivity between brain structures, which may be the origin of psychotic symptoms and cognitive deficits. The consistent findings of ventricular enlargement and decreased brain volume in the hippocampus and cortex may be explained by decreases in dendrites and axons, a hypothesis known as the 'reduced neuropil hypothesis'.[4,8] Thus, it is likely that the underlying cellular mechanisms of the disease comprise developmental changes in the differentiation of dendritic and axonal structures of neurons. Indeed, growth factors, such as brain-derived neurotrophic factor and insulin-like growth factor-1 affecting neuronal differentiation, have a role in schizophrenia.[9,10] Former identified putative susceptibility genes for schizophrenia, such as *NRG1*, *Akt1*, *dysbindin-1* and *DISC-1* have all been implicated in neuronal differentiation and neurite formation in cellular and animal models of neurodevelopment.[8,11–13] Disrupted in schizophrenia 1 (DISC1) is one of the most convincing genetic risk factors for major mental illness identified to date. DISC1 interacts directly with cyclic adenosine mono phosphate (cAMP)-hydrolyzing enzyme phosphodiesterase 4B, an independently identified risk factor for schizophrenia.[14] Moreover, the cAMP signaling pathway is implicated in shaping neuronal morphology during development.[15]

Different lines of evidence have accumulated indicating that neuronal calcium sensor proteins, such as visinin-like-protein-1

[1]Molecular and Cellular Neurosciences Laboratory, Department of Biochemistry and Molecular Biology, Southern Research Institute, Birmingham, AL, USA; [2]Signal Transduction Research Group, Institute of Neurophysiology, Charité University Medicine Berlin, Berlin, Germany and [3]Department of Psychiatry, Ludwig-Maximilians-University, Munich, Germany
Correspondence: Dr KH Braunewell, Molecular and Cellular Neuroscience Laboratory, Department Biochemistry and Molecular Biology, Southern Research Institute, 2000 Ninth Avenue South, Birmingham, AL 35205, USA.
E-mail: braunewell@sri.org
[4]These authors contributed equally to this work.

(VILIP-1; gene name *VSNL1* (visinin-like 1)) and neuronal calcium sensor-1, are associated with schizophrenia. Neuronal calcium sensor proteins are key players in different neuronal signaling pathways[16,17] and are implicated in numerous pathological processes of central nervous system disorders.[18] Recent genome-wide linkage studies in the Korean population have shown association of chromosome 2p24.3 with schizophrenia, where the *VSNL1* gene lies.[19] *VSNL1* has also been correlated with cytoarchitectural abnormalities in schizophrenia and bipolar depression in the US population, with a differentiated gene expression found in the frontal cortex,[20] identifying *VSNL1* as a possible novel susceptibility factor. Earlier data from post-mortem studies in schizophrenic patients and in a ketamine schizophrenia model, revealed already reduced numbers and staining intensity of pyramidal neurons positive for the gene product VILIP-1.[21–23] These results have been confirmed independently by microarray and proteomic studies.[24,25] More recently, increased expression of microRNAs (for example, miR-181b) was discovered in schizophrenia and was found to negatively regulate VILIP-1 expression in schizophrenic brains and in human dopaminergic SH-SY5Y neurons,[26] further adding to evidence implicating VILIP-1 in schizophrenia. Interestingly, insulin and IGF-1, which have also been associated with schizophrenia, increases the expression of genes that show pathological decrease in expression in schizophrenia, including VILIP-1 expression, in human SH-SY5Y model neurons.[27]

Originally, *VSNL1*, which is highly conserved between chick, mouse and human, was identified during a screen for developmentally upregulated genes in the chick visual cortex, where it shows enhanced expression levels during neuronal differentiation and axonal pathfinding.[28] *In vitro* assays have shown that the protein upregulates cAMP levels and differentiation in various peripheral cells, such as pancreatic, kidney, skin and glioma cells.[29–32] Interestingly, in mouse β-cells, overexpression of VILIP-1 increases cAMP, which is accompanied by increased cAMP-responsive element-binding protein gene expression.[31] Moreover, in squamous cell carcinoma, VILIP-1 inhibits rhoA via elevating cAMP levels.[32] The cAMP pathway and cAMP-responsive element-binding protein activation, as well as inhibition of the rhoA-ROCK pathway, have been implicated in promotion of neurite outgrowth and functional recovery of neurons.[15,33] Thus, we speculate that VILIP-1, in conjunction with the cAMP pathway, has a role for neuronal differentiation.

The specific aims of the study were to evaluate whether *VSNL1* single-nucleotide polymorphisms (SNPs) show association with schizophrenia, and especially associate with one of the most sensitive measures for assessing impaired frontal cortical function, the Wisconsin Card Sorting Test (WCST).[34] We next asked what the functional impact of *VSNL1* SNPs and pathological expression of VILIP1 might be for neurodevelopment. Therefore, we have investigated whether VILIP-1 can influence cAMP signaling in neurons and have started to decipher its effect on neuronal morphology.

Materials and methods

Materials. Recombinant nerve growth factor and siRNA for VILIP-1 and control small interfering RNA (siRNA) were obtained from Invitrogen (Carlsbad, CA, USA). Forskolin, DDA (2′,5′-dideoxyadenosine), H89 and KT5720 were from Tocris Biosciences (Ellisville, MO, USA) or from Sigma (St Louis, MO, USA). SMI-312 was from Covance (Austin, TX, USA). Affinity purified antibody against VILIP-1 was described previously.[35]

Cell culture and transfection of hippocampal neurons. Hippocampal cultures were prepared from fetal wistar rat brains (E18) and cultured as previously described.[35] Cells were plated onto poly-D-lysin coated 12-well culture plates at a density of 120 000 cells for transient transfection experiments and onto 24-well culture plates with glass coverslips at a density of 60 000 cells for immunocytochemistry. After 24 h of plating, culture medium was exchanged for neurobasal medium/b27 medium (Invitrogen), and hippocampal neurons were transfected after 1 day in culture with complementary DNA for green fluorescent protein (GFP), VILIP-1-GFP[35] or siRNA, using the lipofectamin transfection method (Invitrogen) and cultured for 48 h.

Cell culture and transfection of SH-SY5Y cells. SH-SY5Y cells were from ATCC (ATCC Number: CRL-2266, Salt Lake City, UT, USA) and cultured according to the suppliers instructions. For neurite outgrowth assays, 9 000 SH-SY5Y cells were placed in each 24-well cell culture plate with media containing 10% fetal bovine serum for 24 h, followed by neurobasal/B27 medium. After 4 h of incubation, different chemicals were added to study the specific pathways involved (nerve growth factor and KT5720, 100 nM; DDA, 300 μM) for an additional 48 h. The Amaxa nucleofector system (Lonza, Cologne, Germany) was used for transfection with GFP and GFP-VILIP-1, or siRNA knockdown experiments with siRNA for VILIP-1 and scrambled control siRNA. Transfection efficiency was measured by counting percentage of cells showing green fluorescence, using a confocal microscope (TCS SPE, Leica Microsystems, Buffalo Grove, IL, USA).

Cell morphology analysis. Photographs of hippocampal neurons and SH-SY5Y neurons were analyzed with the Image J software (version1.41, NIH, http://rsb.info.nih.gov/ij/index.html) using the Sholl and neuron J plugin. For Sholl-analysis, quantification of transfected hippocampal neurons was performed by counting the number of neurite intersections of concentric circles placed around the center of the cell soma in gradually increasing radii from 20 to 100 μm. For SH-SY5Y cells, number and length of neurites longer than the diameter of one cell were measured using the neuron J plugin.

Western blot analysis. Hippocampal neurons (60 000) or SH-SY5Y cells (10 000) were cultured in 12-well cell culture plates for western blot analysis. After 48 h of transfection, the cells were lysed, and western blot analysis using primary anti-VILIP-1 antibody or anti-actin antibody for control was performed as previously described.[35]

Cyclic AMP assay. The production of cyclic adenosine mono phosphate (cAMP) was measured using the Amersham enzyme immunoassay kits (EIA 225, Amersham, Uppsala, Sweden) or the Cisbio HTRF c-AMP kit (Cisbio, Bedford, MA, USA) for hippocampal neurons and SH-SY5Y neurons, respectively. Transfected cells (60 000 hippocampal neurons, 30 000 SH-SY5Y neurons) were analyzed following the manufacturers' protocols.

Schizophrenic patients. A total of 627 individuals with schizophrenia were ascertained from the Munich area in Germany. Of them, 66% were of German descent (that is, both parents were German) and 34% were other Caucasians from middle Europe. Case participants had a DSM-IV and International Classification of Disease-10 (ICD-10) diagnosis of schizophrenia with the following subtypes: paranoid 78%, disorganized 15%, catatonic 2% and undifferentiated 5%. Detailed medical and psychiatric histories were collected, including the Structured Clinical Interview for DSM-IV (SCID), to evaluate lifetime axis I and II diagnoses.[36,37] All details of this group of schizophrenic patients have been published elsewhere.[38] Four physicians and one psychologist rated the SCID interviews and all measurements were double rated by a senior researcher. Exclusion criteria included a history of head injury or neurological diseases. All case participants were outpatients or stable inpatients.

Healthy controls. A total of 541 unrelated volunteers of German descent (that is, both parents German) were randomly selected from the general population of Munich, Germany, and contacted by mail. To exclude subjects with central neurological diseases and psychotic disorders or subjects who had first-degree relatives with psychotic disorders, several screenings were conducted before the volunteers were enrolled in the study. First, subjects who responded were initially screened by phone for the absence of neuropsychiatric disorders. Second, detailed medical and psychiatric histories were assessed for both themselves and their first-degree relatives by using a semi-structured interview. Third, if no exclusion criteria were fulfilled, they were invited to a comprehensive interview including the SCID I and SCID II[36,37] to validate the absence of any lifetime psychotic disorder. Additionally, the Family History Assessment Module[39] was conducted to exclude psychotic disorders among their first-degree relatives. Furthermore, a neurological examination was conducted to exclude subjects with current central nervous system impairment. In the case that the volunteers were older than 60 years, the Mini-Mental Status Test[40] was performed to exclude subjects with possible cognitive impairment. Written informed consent was obtained from all subjects after a detailed and extensive description of the study, which was approved by the local ethics committee and carried out in accordance to the ethical standards laid down in the Declarations of Helsinki.

Wisconsin card sorting test. A subset of the patients and controls were further characterized. A total of 566 (353 healthy volunteers, 213 patients) subjects participated in the WCST (Computer Version 4),[41] where cards have to be sorted against a set of four standard cards according to a certain unknown rule, such as color, shape or number of symbols on the cards. Sorting rules changed according to predefined criteria.

Genotyping. DNA extraction was done with the QIAamp Blood Maxi Kit (QIAamp DNA Blood Midi/Maxi Handbook, Firma Qiagen, Hilden, Germany, 2005). DNA concentration was adjusted using the PicoGreen quantitation reagent (Invitrogen, Karlsruhe, Germany). The samples were genotyped using the Illumina HumanHap300 BeadChip according to the manufacturer's specifications (Illumina, San Diego, CA, USA). All solutions and reagents are from Illumina and were used according to the manufacturer's specifications. Several quality checks for genome-wide studies, like check for duplicates, check for gender or check for batch clusters were performed (for details see Stefansson et al.[42]). 266.382 SNPs passed the quality control in 1168 individuals with the following filters: (i) individual call rate $>95\%$, (ii) SNP call rate $>99\%$, (iii) minor allele frequency <0.01, (iv) deviation from Hardy–Weinberg equilibrium in controls at $P<10E-06$. After removing eight individuals, no population stratification was seen. For the chromosomal region of interest in this paper, SNPs were selected between the positions (Chr 2: 17518961 and 17665665) covering eight SNPs. Only samples with a call rate greater than 98% were included in the analysis. The Hardy–Weinberg equilibrium was $P>0.05$ in all SNPs and subsamples.

Statistical analysis. Significance of the neurite outgrowth results was analyzed using Microsoft excel and Insat software. Average neurite length was expressed as mean $\pm$ s.e. One-way analysis of variance was used to evaluate significant differences between treatment conditions. Sociodemographic parameters such as χ^2-, t-tests or analysis of variance were used as appropriate. For case–control studies, an additive model in PLINK was used.[43] For analyses of WCST, the covariates 'age', 'gender' and 'education', as well as the 'disease status' in the whole sample were included. Again, an additive model was calculated. Haploview 4.0 (http://www.broad.mit.edu/mpg/haploview/) was used to generate a linkage disequilibrium map (r2 and D', see Supplementary Figure 1). Tests for associations using multimarker haplotypes were performed using the software environment 'R' (http://www.R-project.org). Global and single haplotype significance and odds ratios were calculated. Nominally significant asymptotic P-values were confirmed by permuting the case–control status over 10 000 replicates to adjust for multiple testing and observing the maximum test statistic in each case. To compare quantitative traits between the haplotypes, the R software package 'haplo.score' was used, integrating the covariates sex, age, education and diagnosis as appropriate. All nominally significant P-values were confirmed by permuting over 10 000 replicates and observing the maximum test statistic (simulated P-value; sim.p).

Results

Association of *VSNL1* SNPs with schizophrenia. Here we report for the first time, an association of SNPs in the *VSNL1* locus (protein name VILIP-1) with schizophrenia. Two intergenic SNPs, rs394874 and rs424827, located on chromosome 2p24.3 (17518961, 17577281), 5′ of the *VSNL1* gene encoding C/T and A/G polymorphisms, and all other intragenic SNPs (rs650275, rs834504, rs1519477, rs12470654, rs1033297 and rs11677051; chromosomal location: 17623649, 17665665) showed significant association ($P = 0.0080$, $P = 0.0097$, $P = 0.0178$, $P = 0.0190$, $P = 0.0210$, $P = 0.0054$, $P = 0.0384$ and $P = 0.0053$, respectively, Table 1) with schizophrenia. Regarding linkage disequilibrium, one block of all eight SNPs was detected with high to moderate D′ (Supplementary Figure 1). Haplotype analysis could further confirm the case–control results, showing one haploytpe with a higher frequency in schizophrenia patients (CGTCTAAG, frequency cases versus controls = 27.7 versus 24.0%, $\chi^2 = 4.034$, $P = 0.0446$) and the opposite protective haplotype less frequent in patients (TACTCGGA, frequency cases versus controls = 23.7 versus 28.2%, $\chi^2 = 6.155$, $P = 0.0131$, Table 2). These data identify *VSNL1* as a novel putative schizophrenia susceptibility gene.

Association of *VSNL1* SNPs with WCST in schizophrenia. The WCST measures higher executive, so-called frontal lobe functions, and perseverative errors have been shown to correlate positively with the occurrence of schizophrenia.[34] For two of the intragenic SNPs, rs12470654 and rs11677051, a replicated association for the number of completed categories (controls/patients/combined: rs11677051: $P = 0.0029$/$P = 0.0321$/$P = 0.0003$; rs12470654: $P = 0.0442$/$P = 0.0247$/$P = 0.0028$), as well as for the schizophrenia-specific perseverative error was found in the WCST (rs11677051: $P = 0.0095$/$P = 0.0180$/$P = 0.0004$; rs12470654: $P = 0.0401$/$P = 0.0141$/$P = 0.0012$), with a lower performance in rs11677051 A-carriers and in rs12470654 in G-carriers. To avoid multiple testing problems, only associations, which replicated in both groups with the same risk allele (healthy controls and schizophrenia patients) as well as in the combined group, are presented here (for all data see Supplementary Material, Table 1). These results indicate that *VSNL1* may be implicated in cognition and specifically in frontal cortex-dependent cognitive impairments in schizophrenia.

Effect of modulation of VILIP-1 expression on cAMP signaling in hippocampal neurons. To look into the possible biological significance of the association of *VSNL1* SNPs with the occurrence of schizophrenia, we evaluated the possible effect of pathologically altered expression of its gene product VILIP-1 on neuronal differentiation. We first examined localization of a heterologously expressed VILIP-1-GFP construct in hippocampal neurons. Fluorescence microscopy revealed localization throughout hippocampal neurons in soma and all processes (Figures 1a and b). Accumulation in some hot spots along neurites and presumably neuritic growth cones is observed (Figure 1a, asterisks).

Table 1 Association of *VSNL1* SNPs with schizophrenia

SNP	POS	LOC	A1	A2	Controls (n)	Patients (n)	All (n)	Controls (MAF)	Patients (MAF)	All (MAF)	Allele	P-values	OR
rs394874	17518961	Intergenic	C	T	191|271|179	269|281|174	460|552|153	0.604|0.396	0.656|0.344	0.632|0.368	T	0.0080	0.7934
rs424827	17577281	Intergenic	A	G	78|272|191	72|288|267	150|560|458	0.396|0.604	0.344|0.656	0.368|0.632	A	0.0097	0.797
rs650275	17586135	VSNL1	C	T	301|203|137	305|266|155	606|469|192	0.744|0.256	0.7|0.3	0.72|0.28	T	0.0178	1.247
rs834504	17588756	VSNL1	C	T	37|203|301	55|266|306	92|469|607	0.256|0.744	0.3|0.7	0.28|0.72	C	0.0190	1.244
rs1519477	17592796	VSNL1	C	T	301|203|137	306|267|154	607|470|191	0.744|0.256	0.701|0.299	0.721|0.279	T	0.0210	1.24
rs12470654	17623649	VSNL1	A	G	188|270|183	264|290|173	452|560|156	0.597|0.403	0.652|0.348	0.627|0.373	G	0.0054	0.7844
rs1033297	17632093	VSNL1	A	G	35|194|312	51|252|324	86|446|636	0.244|0.756	0.282|0.718	0.265|0.735	A	0.0384	1.215
rs11677051	17665665	VSNL1	A	G	63|224|252	44|252|331	107|476|583	0.325|0.675	0.271|0.729	0.296|0.704	A	0.0053	0.7771

Abbreviations: SNP, single-nucleotide polymorphism; POS, position of the SNP on the chromosome; LOC, location of the SNP on the chromosome; A1, A2, alleles 1 and 2; n, number of controls, patients and the combined group; MAF, minor allele frequency.
P-value for case–control comparison under the additive model.

Costaining with the dendritic marker microtubule-associated protein 2 reveals that VILIP-1 is present in dendrites as well as in microtubule-associated protein 2-negative, axonal extensions (Figure 1b), similar to our previous observations.[35] To test whether VILIP-1-GFP expression may affect neuronal signaling and, in turn, neuronal differentiation of hippocampal neurons, we transfected E19 hippocampal neurons before differentiation *in vitro* with GFP-VILIP-1 and knocked down expression with VILIP-1 siRNA, and monitored expression by western blot analysis (Figures 1c and d). VILIP-1-GFP transfection produced an additional VILIP-1-positive protein band at an molecular weight of 50 kDa above the endogenous 22-kDa band (Figure 1c), whereas VILIP-1 siRNA knockdown reduced the expression of endogenous VILIP-1 by about 45% (Figures 1c and d). Next, the effect of VILIP-1-GFP overexpression or VILIP-1 knockdown on forskolin-stimulated adenylyl cyclase activity was evaluated (Figure 1e). A significant increase of cAMP levels following VILIP-1-GST transfection, and a decrease following siRNA knockdown of VILIP-1 expression indicated that VILIP-1 affects cAMP signaling in hippocampal neurons.
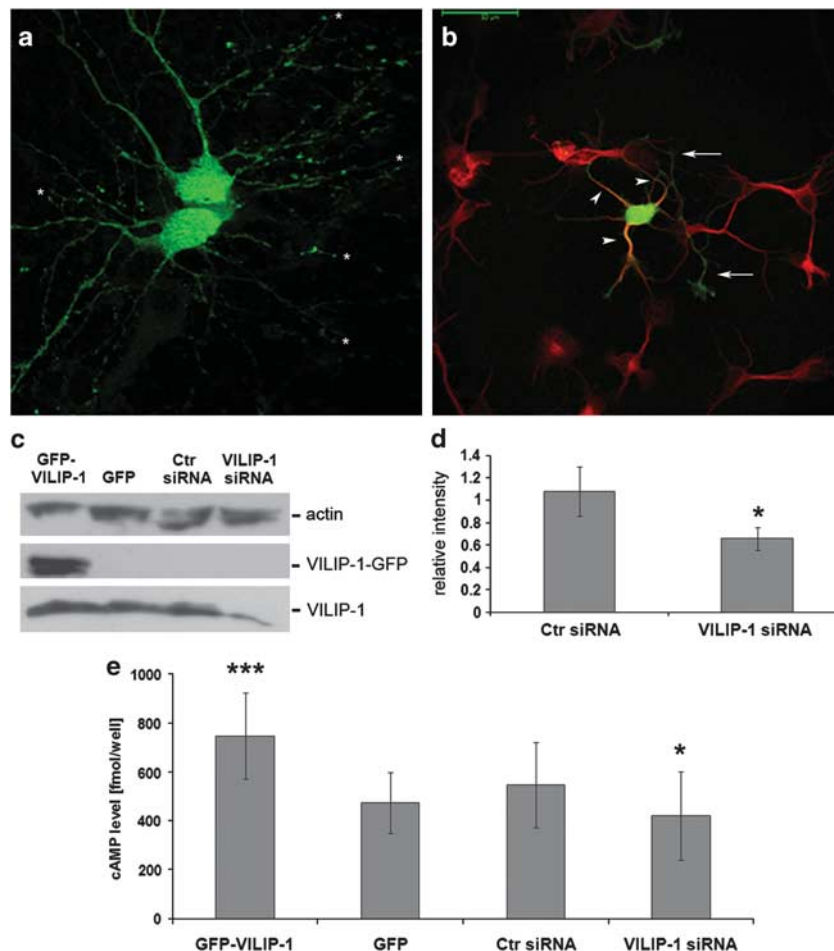
Table 2 Association of *VSNL1* SNP haplotypes with schizophrenia

Block	Frequencies (%) cases, controls	χ^2-test	P-values
CGCTCAGG	0.327, 0.311	0.711	0.3991
CGTCTAAG	**0.277, 0.240**	**4.034**	**0.0446**
TACTCGGA	**0.237, 0.282**	**6.155**	**0.0131**
TACTCGGG	0.086, 0.088	0.010	0.9206
CGCTCGGA	0.022, 0.031	2.114	0.1460
TACTCAGG	0.018, 0.023	0.670	0.4129
CGTCTAGG	0.018, 0.013	1.040	0.3079

Abbreviations: SNP, single-nucleotide polymorphisms; *VSNL1*, visinin-like 1. Haplotypes of the eight studied SNPs and their *P*-values for case–control association are presented. Haplotypes with a frequency <1% were excluded. Significant haplotypes are in bold.

Figure 1 Visinin-like-protein-1 (VILIP-1)-green fluorescent protein (GFP) overexpression and small interfering RNA (siRNA) knockdown of endogenous VILIP-1 expression in hippocampal neurons. (**a**) Fluorescence detection of VILIP-1-GFP expression in two neighboring hippocampal neurons in culture. Note, hot spots of expression in fine neuritic extensions and growth cones (asterisks). (**b**) Localization of VILIP-1-GFP in axons and dendrites in VILIP-1-GFP-transfected (green) and non-transfected neurons costained with the dendritic marker microtubule-associated protein 2 (red). Note, co-staining in yellow is seen in dendritic structures (arrowheads), whereas only green fluorescence represents VILIP-1-GFP expression in axons (arrows). (**c**) Western blot analysis monitoring endogenous and VILIP-1-GFP overexpression (V1-GFP) and siRNA knockdown of endogenous VILIP-1 expression in hippocampal neurons. Immunostaining of actin serves as loading control. (**d**) Quantification of the effect of VILIP-1 siRNA knockdown (siRNA) of endogenous VILIP-1 expression. (**e**) Effect of VILIP-1-GFP overexpression (V1-GFP) compared with GFP control (empty), and of siRNA knockdown of endogenous VILIP-1 (siRNA) compared with scrambled siRNA control (scr) on forskolin-induced cAMP-accumulation in hippocampal neurons. Mean values from at least three independent experiments ($n = 3$) are shown and error bars indicate s.d. Asterisks indicate significant (*) and highly significant (***) differences of the mean values.

Effect of modulation of VILIP-1 expression on morphological differentiation of hippocampal neurons. When hippocampal cultures transfected with VILIP-1-GFP and GFP vector alone were compared, obvious changes in neuronal morphology of transfected cells were observed 48 h following transfection (Figures 2a and b). Compared with GFP control transfection, VILIP-1-GFP transfection led to a noticeable stronger differentiation with a higher degree of branching (Figure 2, compare A with B). For quantification purposes, we aimed to identify the transfected neurons only, as hippocampal neurons show relatively low transfection efficiences. Therefore, we co-transfected a GFP construct with siRNA against VILIP-1 or the control siRNA (Figures 2c and d). Only in the case of GFP-co-transfected neurons containing VILIP-1 siRNA, the expression of endogenous VILIP-1 was diminished (Figure 2c) compared with GFP/control siRNA co-transfected neurons (Figure 2d), as revealed by co-immunostaining against VILIP-1 protein (in red). Quantification of transfected hippocampal neurons was performed by Sholl analysis, which is a simple and established tool to characterize the morphological characteristics of neurons.[44] Fluorescence images from GFP, VILIP-1-GFP, GFP/VILIP-1 siRNA and GFP/control siRNA-co-transfected hippocampal neuronal cultures, we analyzed using the half-automatic Sholl–Plugin for NIH ImageJ software. The number of dendrite intersections of concentric circles around the center of the cell soma was counted in gradually increasing radii from 20 to 100 µm (Figure 3a). Overexpression of VILIP-1-GFP leads to increased number of dendritic arborization close to
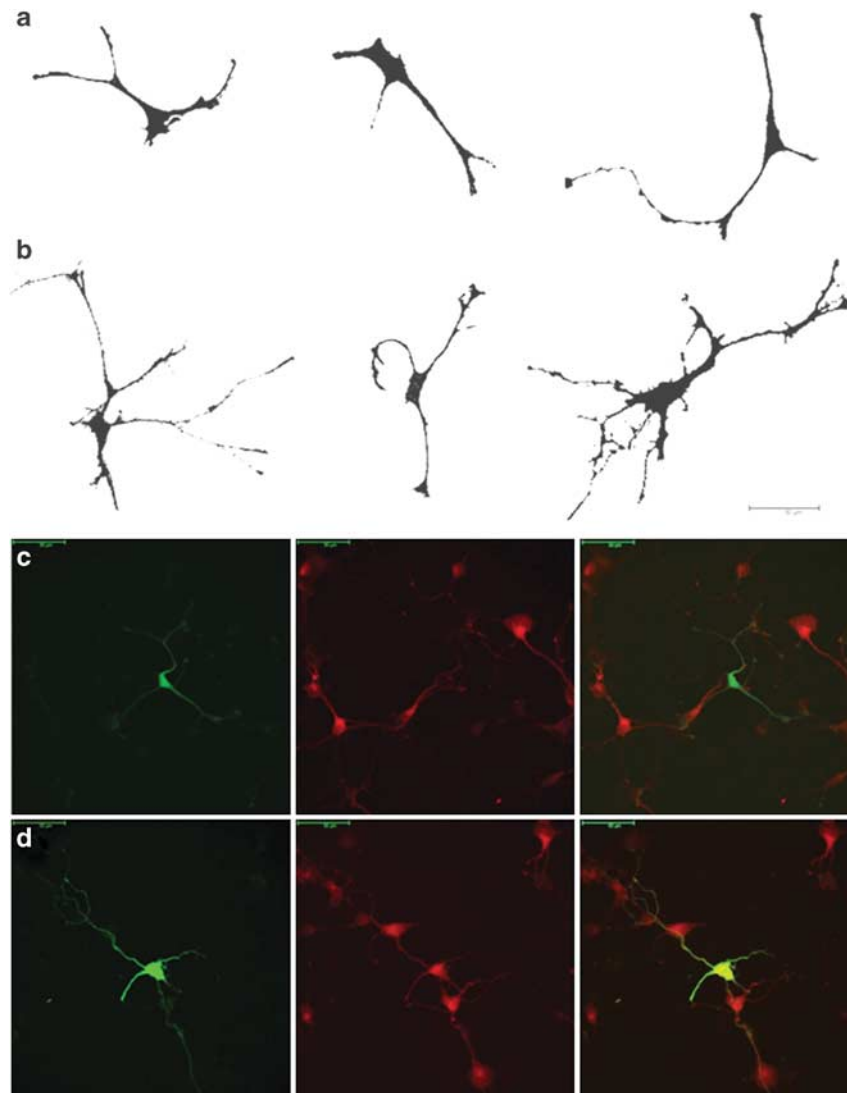

Figure 2 Effect of visinin-like-protein-1 (VILIP-1)- green fluorescent protein (GFP) on neuronal morphology and silencing of VILIP-1 expression with small interfering RNA (siRNA) in hippocampal cultures. (**a** and **b**) Examples of dendritic morphologies of GFP (**a**) versus VILIP-1-GFP (**b**) transfected hippocampal neurons 48 h following transfection. Bar in B is 50 µm. (**c** and **d**) Silencing of VILIP-1 expression in hippocampal cultures with siRNA. Confocal fluorescence pictures GFP (green) and anti-VILIP-1- or control-siRNA co-transfected (after 1 div) hippocampal neurons (3 div) immunostained with anti-VILIP-1/Cy3 antibodies (red). (**c**) Shows anti-VILIP-1-siRNA-transfected, whereas (**d**) shows control-siRNA-transfected hippocampal neurons. The first column shows the green GFP channel, column 2 the red immunostaining channel for VILIP-1/Cy3, and column 3 depicts the overlay of channel 1 and 2. Regions of colocalization of immunofluorescence appear yellow. Bars are 50 µm.
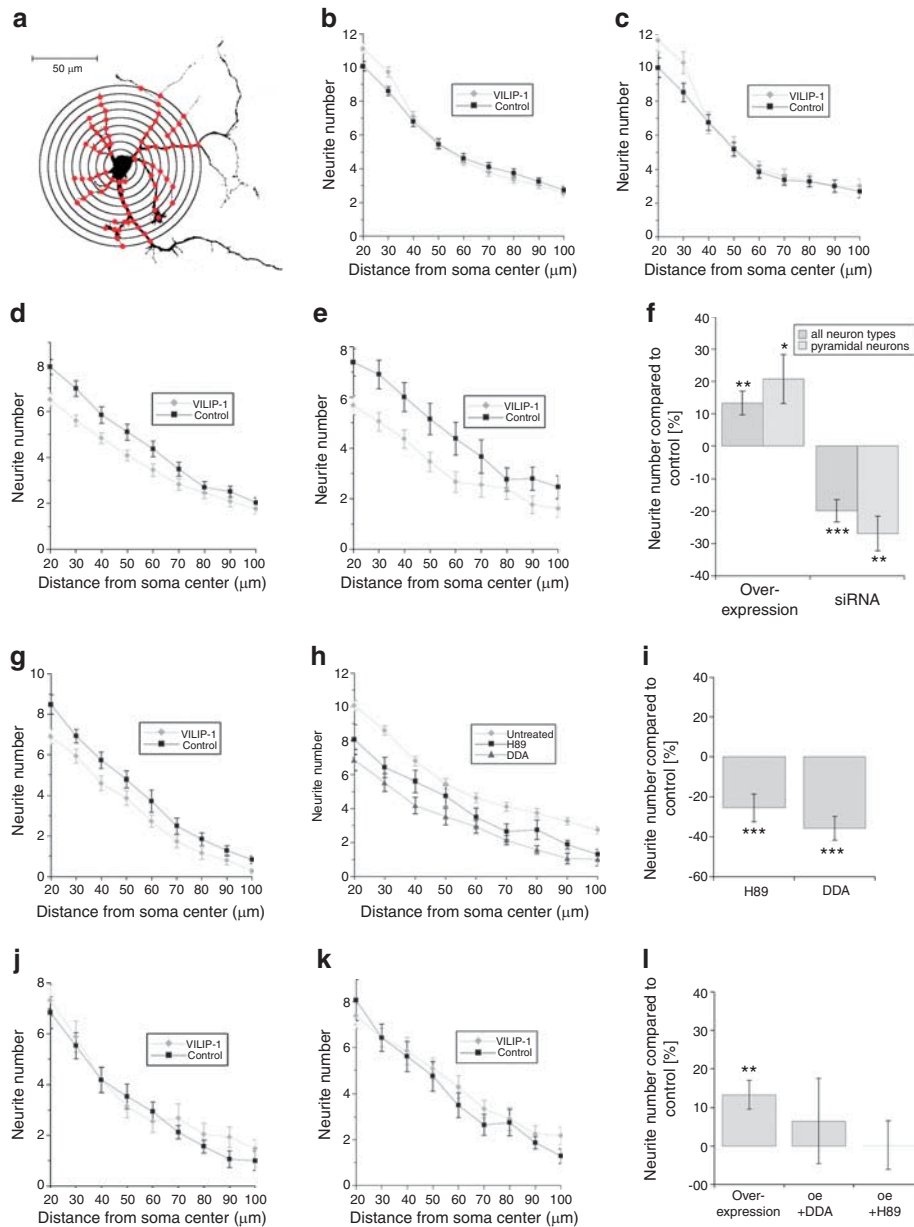
Figure 3 Effect of visinin-like-protein-1 (VILIP-1) expression and cyclic adenosine monophosphate (cAMP) signaling on branching in hippocampal neurons. (**a**) Exemplification of the Sholl analysis showing counting of neurites in concentric rings (20–100 μm) around the cell soma of a hippocampal neuron as a measure for dendritic arborization. Bar in A is 50 μm. (**b**) Effect of VILIP-1-green fluorescent protein (GFP) overexpression (VILIP-1) on the number of dendrites in the distance from 20 to 100 μm from the cell soma in all neurons. (**c**) Effect of VILIP-1-GFP overexpression on the number of dendrites in the distance from 20 to 100 μm from the cell soma in pyramidal neurons. (**d**) Effect of VILIP-1-knockdown (VILIP-1) on the number of dendrites in the distance from 20 to 100 μm from the cell soma in all neurons. (**e**) Effect of VILIP-1 knockdown on the number of dendrites in the distance from 20 to 100 μm from the cell soma in pyramidal cells. (**f**) Summary of the effect of VILIP-1-GFP overexpression and VILIP-1 small interfering RNA (siRNA) knockdown on the number of dendrites in 30 μm distance from the soma center in all neuron types and in pyramidal neurons. Data were from 100 neurons (25 for pyramidal neurons) per group out of three experiments. Asterisks indicate significant (*), high significant (**), and highly significant (***) differences of the mean values. (**g-l**) Effect of enhanced and reduced cAMP-signaling, and cAMP-dependent effect of VILIP-1 on branching in hippocampal neurons. (**g**) Effect of forskolin on the number of dendrites in the distance from 20 to 100 μm from the cell soma in all neurons compared with control. (**h**) Effect of H89 and 2′,5′-dideoxyadenosine (DDA) on the number of dendrites in the distance from 20 to 100 μm from the cell soma compared with control. (**i**) Summary effect of DDA and H89 on the number of dendrites in the distance from 20 to 100 μm from the cell soma in hippocampal neurons compared with control. (**j**) Effect of H89 on the VILIP-1-GFP-dependent increase in the number of dendrites in the distance from 20 to 100 μm from the cell soma in all neurons. (**k**) Effect of DDA on VILIP-1-GFP-dependent increase in number of dendrites in the distance from 20 to 100 μm from cell soma in all neurons. (**l**) Summary of the effect of DDA and H89 treatment on the VILIP-1-GFP-dependent increases in the number of dendrites in the distance from 20 to 100 μm from the cell soma in all neurons. Data were from 100 neurons (25 for pyramidal neurons) per group out of three experiments.

the soma (Figures 3b and c). The greatest effect of VILIP-1 expression is observed at 30 μm distance from the soma center when all neurons were analyzed (Figure 3b) and in

neurons with typical pyramidal morphology (Figure 3c). When treatment with siRNA against VILIP-1 was compared with control siRNA treatment, significant differences in

dendritic morphology was observed over a wider range of distance. VILIP-1 led to a significant decrease in the number of dendrites between 20 and 60 μm distance from the cell soma in all neurons and in neurons with pyramidal phenotype (Figures 3d and e). Quantification of the data at 30 μm distance from the soma center showed a significant increase of 13% when all neurons were analyzed and a 21% increase in neurons with typical pyramidal morphology following VILIP-1-GFP transfection. siRNA for VILIP-1 produced 20% decrease in neurite number in all neurons versus 27% decrease for pyramidal cells (Figure 3f). These results indicate that VILIP-1 overexpression leads to formation of novel neurites close to the soma, whereas reduced VILIP-1 expression leads to a general reduction in the number of neurites at all distances from the soma.

Effect of VILIP-1 expression on morphological differentiation of hippocampal neurons depends on cAMP signaling. Next, we analyzed the causal correlation between the effect of VILIP-1 on cAMP production and its effect on dendritic arborization. As control treatment to show that cAMP affects neurite growth in the hippocampal culture system, forskolin was applied to enhance cAMP accumulation, and the general adenylyl cyclase inhibitor DDA and the protein kinase A inhibitor H89 were applied to reduce cAMP signaling in hippocampal neurons. As expected, these treatments led to enhancement and reduction of the number of dendrites, respectively (Figures 3g and h). Quantification of the effect revealed that the two inhibitors, H89 and DDA, significantly reduced neurite outgrowth by 25 and 35%, respectively (Figure 3i). When VILIP-1-GFP-transfected hippocampal cultures were treated with the two inhibitors of the cAMP pathway, DDA and H89, the VILIP-1-dependent increase in number of dendrites was completely abolished (Figures 3j and k). Quantification of the effect revealed that the inhibitors significantly reduced the VILIP-1-dependent enhancement of dendritic growth to background levels (Figure 3l). Thus, the effect of VILIP-1 on formation of new dendritic processes depends on increased cAMP levels.

Overexpression and siRNA knockdown of VILIP-1 in SH-SY5Y neurons and effect on cAMP signaling. The human neuronal cell line SH-SY5Y has been used as a cellular model for schizophrenia, in which growth factors implicated in schizophrenia lead to the regulation of schizophrenia susceptibility genes such as *VSNL1*.[27] Therefore, we have tested the effect of VILIP-1 on neuronal morphology of SH-SY5Y cells. VILIP-1-GFP expression in SH-SY5Y neurons was shown to be present in axonal as well as dendritic neurites, as revealed by co-staining with the axonal marker SMI-312 (Figures 4a and b). Often, VILIP-1 was found to be enriched in growth cones of dendrites and axons (Figures 4a and c, asterisks). The overexpression of VILIP-1-GFP in SH-SY5Y neurons produced an additional VILIP-1-positive protein band at a molecular weight of 50 kDa above the 22-kDa endogenous protein band (Figure 4a), whereas VILIP-1 siRNA knockdown reduced the expression of endogenous VILIP-1 by 65% (Figures 4a and e). Moreover, VILIP-1-GFP overexpression has a significant effect on

forskolin-stimulated cAMP accumulation, when compared with GFP-control-transfected neurons (Figure 4f). Significantly decreased cAMP levels were observed following siRNA knockdown of VILIP-1 expression (Figure 4g). These results indicate that VILIP-1 affects cAMP accumulation in the human neuronal cell line SH-SY5Y.

Effect of modulation of VILIP-1 expression and cAMP signaling on morphological differentiation of SH-SY5Y neurons. For evaluating the effect of VILIP-1 on dendritic growth, we transfected SH-SY-5Y cells with either GFP vector and VILIP-1-GFP, or with control siRNA and siRNA for VILIP-1, and quantified differences in number and average length of neurites, assuming that with only one axon per neuron, neurites will be mainly of dendritic origin (Figures 4a–c). VILIP-1-GFP overexpression (Figure 5, compare b with a) produces an increase, whereas siRNA treatment (Figure 5, compare d with c) leads to reduction in number and length of neurites as revealed by quantification of the average neurite length (Figure 5e). In addition, we used different chemicals, which are either activators or inhibitors of the cAMP signal pathway, to understand the exact mechanism by which VILIP-1 exerts its effects. The general inhibitor of adenylyl cyclases DDA and the protein kinase A inhibitor KT5720 were able to attenuate the VILIP-1 effect on neurite length in nerve growth factor-treated SH-SY5Y cells (Figure 5e). DDA reduced the average neurite length below control levels, which indicate that other factors besides VILIP-1 contribute to cAMP-dependent neurite extension in nerve growth factor-treated neurons. When additional parameters were analyzed, the number of branch points showed an even more pronounced effect. VILIP-1 increases dendrite branching by more than 100% (Figure 5f). Moreover, VILIP-1 seems to strongly induce formation of filopodia (Figure 5g). The examples show that in comparison with GFP-transfected SH-SY5Y cells, VILIP-1-GFP-transfected neurons show extensive branching, never observed in control cultures. Following quantification, a 100% increase in the number of neurons with extensive filopodia formation is observed (Figure 5h), indicating that VILIP-1 may particularly affect initial formation of filopodia, which typically proceeds neurite formation and dendritic branching.

Discussion

In this study, we have examined a set of SNPs located intragenic and intergenic (5′) of the *VSNL1* gene for association with the occurrence of schizophrenia in a cohort of patients and controls.[38,42] We have identified significant association of two intergenic and six intragenic SNPs with schizophrenia, and two of the intragenic SNPs significantly associated with the WCST.[41] For WCST, only replicated SNPs that were associated in cases only, in controls only and in the combined group with the same risk allele are highlighted. The WCST is used to assess higher cognitive functions, so-called executive functions, such as strategic planning, organized searching, utilizing environmental feedback to shift cognitive sets, directing behavior towards achieving a goal and modulating impulsive responding.[34]
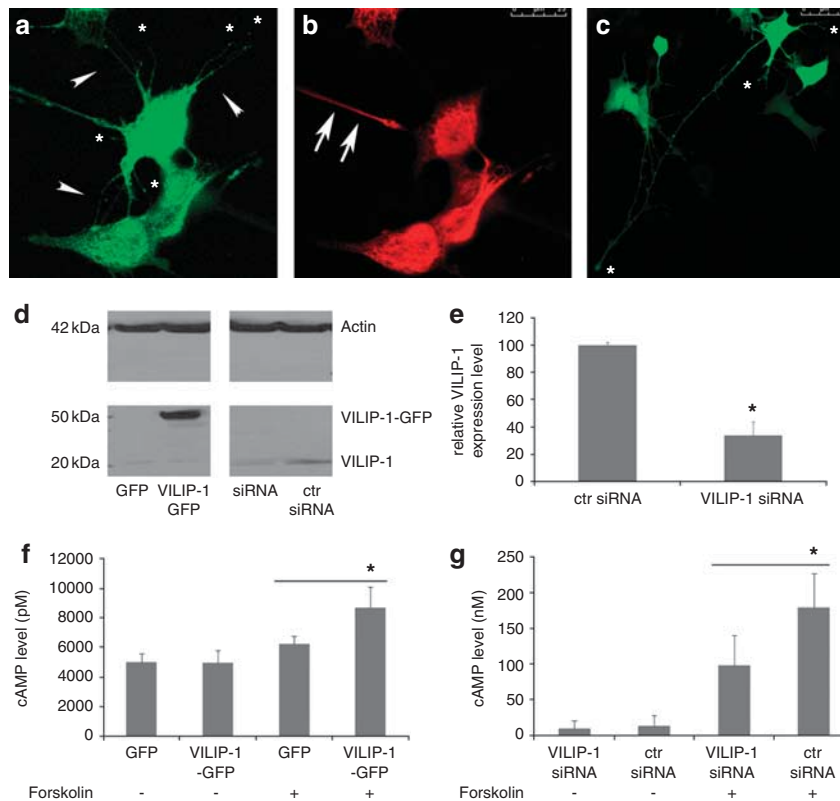
Figure 4 Visinin-like-protein-1 (VILIP-1)-green fluorescent protein (GFP) localization in human SH-SY5Y neurons and effect of VILIP-1-GFP overexpression and small interfering RNA (siRNA) knockdown of VILIP-1 on cyclic adenosine monophosphate (cAMP) accumulation. (**a–c**) Fluorescence picture of (**a**) VILIP-1-GFP transfected neuron, stained with (**b**) the axonal marker SMI-312. Arrow indicates a VILIP-1-GFP- and SMI-312-positive axon, whereas arrowheads indicate VILIP-1-positive, but SMI-312-negative dendrites. Asterisks show growth cones enriched with VILIP-1-GFP fluorescence. (**c**) Example of VILIP-1-GFP-transfected SH-SY5Y neuron with long, probably axonal structure and several dendritic-like neurites. Asterisks mark VILIP-1-GFP enriched growth cones. Bar is 25 μm. (**d–g**) Effect of VILIP-1-GFP overexpression and siRNA knockdown of VILIP-1 on cAMP accumulation in SH-SY5Y neurons. (**d**) Western blot analysis monitoring VILIP-1-GFP overexpression and siRNA knockdown of VILIP-1. Antibodies against VILIP-1 and against GFP stain endogenous VILIP-1 at 22 kDa and a VILIP-1-GFP band at 45 kDa, respectively. Actin immunostaining serves a loading control. (**e**) Quantification of siRNA knockdown of VILIP-1 in SH-SY5Y neurons as shown in (**d**). (**f**) Effect of transfection of SH-SY5Y neurons with VILIP-1-GFP or GFP alone on basal and forskolin-induced cAMP levels. (**g**) Effect of transfection of SH-SY5Y with either VILIP-1 siRNA control or VILIP-1 siRNA on basal and forskolin-induced cAMP levels. Mean values from at least three independent experiments ($n = 3$) are shown and error bars indicate s.d. Asterisks indicate significant (*) differences of the mean values.

The common test relies upon a number of intact cognitive functions including attention, working memory and visual processing. WCST perseverative errors have been shown to correlate positively with lesions of the dorsolateral frontal lobe and schizophrenia, suggesting that deficiencies in conceptual organization and flexibility in abstract thinking occur in patients.[45] In this study, the relationship between the selected SNPs in the *VSNL1* gene and cognitive set-shifting as measured by perseverative errors using the WCST was tested. The results indicate an association of cognitive flexibility and frontally based executive function with *VSNL1* SNPs. However, it is noteworthy that counterintuitively the associated risk alleles for schizophrenia are associated with a better performance in the WCST. Although this finding may have been a surprise, similar results have also been presented; for example, for one of the best replicated risk genes for schizophrenia (*ZNF804A*) there, the schizophrenia allele was associated with better and not lower cognitive performance.[46] Our results are therefore potentially important to our understanding of the genetic mechanisms and pathways that contribute to the susceptibility, as little is known

about the function of *VSNL1*. The aim of our study was therefore to further characterize the functionality of this gene. Especially, as schizophrenia is a very heterogeneous disorder with several subtypes, it is perhaps possible that not all identified susceptibility alleles will have major effects on broad cognitive abilities and that, not all schizophrenia risk alleles will associate with all intermediate phenotypes in further functional studies.

Nevertheless, our results may hint towards an impact of *VSNL1* SNPs and also of altered expression of VILIP-1 in schizophrenia on certain aspects of cognition. It is noteworthy in this context that VILIP-1 was identified as a cerebrospinal fluid biomarker for Alzheimer's disease with association of VILIP-1 levels, with the severity of Alzheimer's disease and association with the Mini-Mental Statement Examination as a measure of cognitive impairment in Alzheimer's disease.[47,48] Future experiments will have to show whether VILIP-1 expression is linked to specific cognitive disturbances, such as attention and/or memory impairment.

Our findings further raise the question of the biological significance of misregulated expression of the novel schizo-
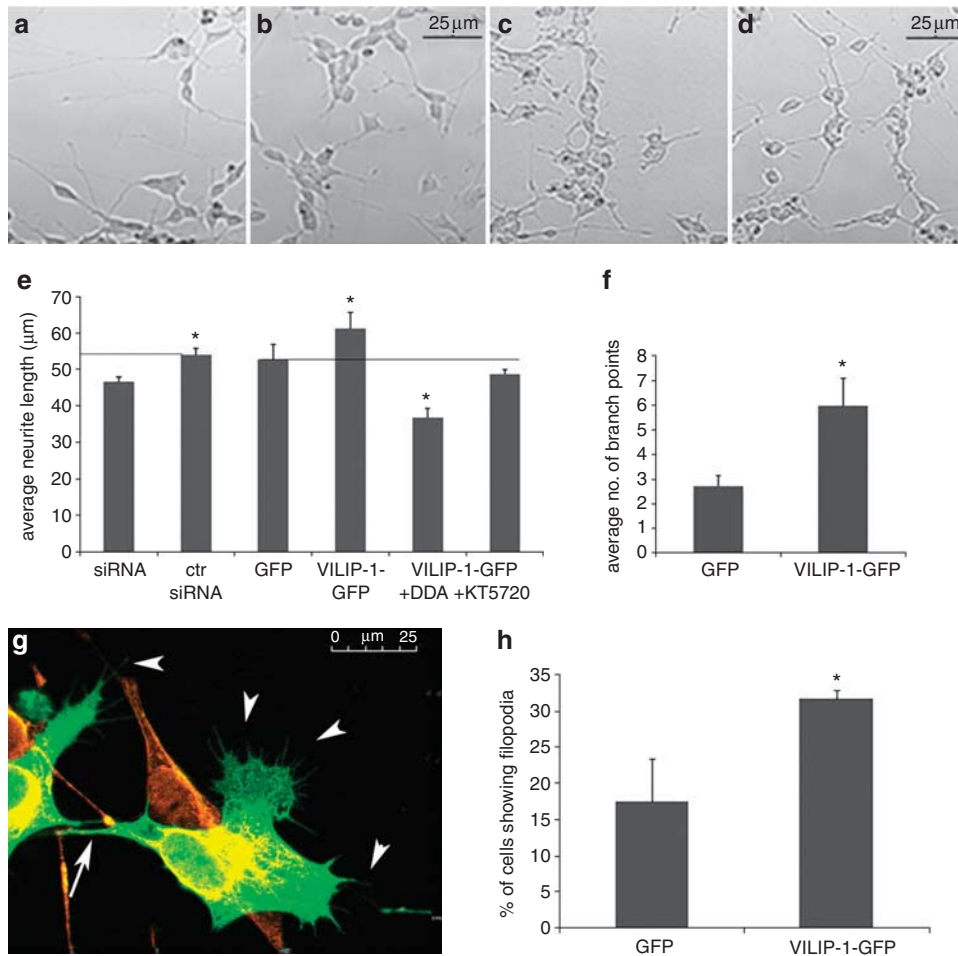
Figure 5 Effect of visinin-like-protein-1 (VILIP-1) expression and cyclic adenosine mono phosphate (cAMP) signaling on neurite outgrowth in nerve growth factor (NGF)-treated human SH-SY5Y neurons. (**a–c**) Phase contrast images of SH-SY5Y neurons transfected with VILIP-1-green fluorescent protein (GFP) (**a**), GFP (**b**), VILIP-1 small interfering RNA (siRNA) (**c**) and siRNA control (**d**). (**e**) Quantification of the effect of VILIP-1- and cAMP signaling on average neurite length of NGF-treated human SH-SY5Y cells. Neurons were treated with VILIP-1 and control siRNA, transfected with GFP and VILIP-1-GFP, and transfected with VILIP-1-GFP and treated with adenylyl cyclase inhibitor $2',5'$-dideoxyadenosine (DDA) or protein kinase A inhibitor KT5720. (**f**) Effect of VILIP-1-GFP transfection on average number of branch points in NGF-treated human SH-SY5Y neurons. Mean values from at least three independent experiments ($n = 3$) are shown and error bars indicate s.d. Asterisks indicate significant (*) differences of the mean values. Bars are 25 μm. (**g**) Example of a neuron extending high numbers of filopodia in NGF-treated VILIP-1-GFP-transfected human SH-SY5Y neurons. (**h**) Quantification of the number of neurons with filopodia in GFP- versus VILIP-1-GFP-transfected cells. Mean values were from at least three independent experiments ($n = 3$) and error bars indicate s.d.. Asterisks indicate significant (*) differences of the mean values. Bar is 25 μm.

phrenia susceptibility factor VILIP-1 during development, which may lead to structural and finally cognitive disturbances in affected brain regions. Therefore, we started to investigate whether upregulation and/or downregulation of VILIP-1 expression during development affect neuronal differentiation, and thus possibly lead to pathological changes in dendritic and axonal structures, which could contribute to developmental disturbances during the course of the disease.[4,5] Interestingly, the schizophrenia susceptibility gene Disc1 has recently been shown to have an effect on neuronal differentiation in that it enhances neurite outgrowth.[14] Disc1 is a physiological binding partner of PDE4, a cAMP-specific phosphodiesterase, thus potentially affecting the cAMP signaling pathway.[15] Previously, VILIP-1 has been shown to affect cAMP-signaling in several heterologous expression systems.[19,20] Reduced cAMP and increased rho-signaling lead to reduced branching of dendritic structures and are both

important signaling system for neuronal differentiation.[15,49] Thus, we have investigated the role of VILIP-1 in conjunction with cAMP-signaling for differentiation of hippocampal neurons and human SH-SY5Y neuronal cells, frequently serving as a cellular model for schizophrenia.[26,27] We were able to show for the first time that VILIP-1 affects cAMP signaling in neuronal cells, and thereby affects cAMP-dependent morphology of neurons. Indeed, cAMP is a well-known promoter of neurite plasticity and branching.[15,33,36,37] However, additional pathways regulating the fine tuning of axonal versus dendritic differentiation and outgrowth, such as GAP43, rhoA and the PIP2/PI3K/AKT/GSK3β pathway also have to be taken into account.[49–53]

One of the main characteristics of neurons is their shape, with every neuron extending long-branched dendrites to create a complex wiring network. Creation of such neuronal network has a central role in neuronal transmission and

central nervous system function. VILIP-1-GFP led to enhanced branching, particularly in short distance from the soma in hippocampal neurons. Similarly, in SH-SY5Y neurons, VILIP-1 strongly induced formation of filopodia and branching. Both results indicate that VILIP-1 might affect already the initial phase of neuritogenesis and branching by enhancing the formation of new filopodia. During early development, filopodia can be transformed into dendritic branches, whereas later in development, filopodial protrusions can develop into dendritic spines.[54,55] Thus, it appears likely that changed expression of VILIP-1 during development of the brain, depending on increased or decreased expression, may positively or negatively affect neuronal outgrowth and particularly, dendritic branching. The elaboration of complex dendritic arbors during development are necessary to integrate synaptic inputs and thus determine proper neuronal network activity.[56] However, it also has been suggested that behavioral symptoms of schizophrenia could arise from over pruning or failed maintenance of dendrites later in life, occurring before and during psychosis development in schizophrenia in late adolescence.[57] It is noteworthy in this context that the modulation of VILIP-1 expression has already been shown to affect neuronal network activity by affecting nicotinergic modulation of GABAergic activity of interneurons in hippocampal cultures.[23] Observed pathological changes in expression levels, up- or downregulation in schizophrenia, for instance as reported in certain types of hippocampal interneurons and in the nucleus accumbens,[22–26] might lead to pathological dendritic morphology of key neuronal subtypes. We hypothesize that this may contribute to subtle changes in neuronal connectivity, leading to some of the cognitive deficits typically measured via tests such as WCST.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Translational Psychiatry website (http://www.nature.com/tp)